# Genome-Wide Analysis of the Arabidopsis Leaf Transcriptome Reveals Interaction of Phosphate and Sugar Metabolism[1][W]

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Global gene expression was analyzed in Arabidopsis (*Arabidopsis thaliana*) by microarrays comprising 21,500 genes. Leaf segments derived from phosphorus (P)-starved and P-replenished plants were incubated with or without sucrose (Suc) to obtain tissues with contrasting combinations of P and carbohydrate levels. Transcript profiling revealed the influence of the two factors individually and the interactions between P- and sugar-dependent gene regulation. A large number of gene transcripts changed more than 2-fold: In response to P starvation, 171 genes were induced and 16 repressed, whereas Suc incubation resulted in 337 induced and 307 repressed genes. A number of new candidate genes involved in P acquisition were discovered. In addition, several putative transcription factors and signaling proteins of P sensing were disclosed. Several genes previously identified to be sugar responsive were also regulated by P starvation and known P-responsive genes were sugar inducible. Nearly 150 genes were synergistically or antagonistically regulated by the two factors. These genes exhibit more prominent or contrasting regulation in response to Suc and P in combination than expected from the effect of the two factors individually. The genes exhibiting interactions form three main clusters with different response patterns and functionality of genes. One cluster (cluster 1) most likely represents a regulatory program to support increased growth and development when both P and carbohydrates are ample. Another cluster (cluster 3) represents genes induced to alleviate P starvation and these are further induced by carbohydrate accumulation. Thus, interactions between P and Suc reveal two different signaling programs and novel interactions in gene regulation in response to environmental factors. cis-Regulatory elements were analyzed for each factor and for interaction clusters. PHR1 binding sites were more frequent in promoters of P-regulated genes as compared to the entire Arabidopsis genome, and E2F and PHR1 binding sites were more frequent in interaction clusters 1 and 3, respectively.

Phosphorus (P) is an essential macronutrient and efficient acquisition of phosphate ($P_i$) is an important factor for plant growth. Consequently, plants have evolved a wide range of morphological and molecular adaptations to increase remobilization, uptake, and efficient use of $P_i$ when availability is low. These adaptations require the plant to sense the level of $P_i$ and change the expression of a number of genes accordingly.

In Arabidopsis (*Arabidopsis thaliana*), such P-responsive genes are exemplified by genes coding for RNases (Bariola et al., 1994), phosphatases (del Pozo et al., 1999), and high-affinity $P_i$ transporters, which are responsible for P uptake against a steep concentration gradient. To some extent, low-affinity $P_i$ transporters are also induced in response to P limitation (Muchhal et al., 1996; Muchhal and Raghothama, 1999; Mudge et al., 2002). Other typical markers for P deficiency are the genes *At4* (Burleigh and Harrison, 1999) and *IPS1* (Martin et al., 2000), which appear to have regulatory functions (Shin et al., 2006). Although numerous P starvation-inducible genes have been reported (Raghothama, 1999), understanding of transcriptional regulation in response to P deficiency is still far from complete and molecular mechanisms that initially sense and transmit information on P status in plants are largely unknown.

The use of microarray techniques in transcriptome analysis has opened new possibilities to elucidate the sensing, signaling, and regulatory pathways of the P starvation response. In a partial transcriptome analysis of responses to $P_i$ starvation in Arabidopsis (Wu et al., 2003), 6,172 genes were analyzed and 29% of the genes were altered in the root and/or shoot. The regulated genes could be divided into several groups according to the temporal pattern of expression and the tissue in which genes were induced or repressed. In another transcriptome study, Hammond et al. (2003) concluded that at least two transcriptional programs

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operate in response to P starvation. First, a transient change in expression during the early stages of starvation preferentially involves genes characteristic for general stress responses. Thereafter, transcriptional changes are induced for genes that mainly play a more specific role in the alleviation of $P_i$ starvation. The concept of a less specific early response and a more specific later response to $P_i$ starvation was also supported by transcriptome analysis of tomato (*Lycopersicon esculentum*) roots following $P_i$, potassium (K), and iron (Fe) limitation (Wang et al., 2002), where expression of several of the early responding genes was coordinately induced by all these types of nutrient stress.

Recently, comprehensive examination of global gene expression in response to P deficiency revealed coordinated induction of 612 genes and suppression of 254 genes, respectively (Misson et al., 2005). The regulated genes were involved in a wide range of functional groups related to metabolism, ion transport, signal transduction, transcriptional regulation, and growth and development. Such results may contribute to the identification of key molecular determinants for the improvement of $P_i$ economy.

Evidence for close interactions between P- and sugar-sensing pathways has recently emerged. Aside from the crucial role of P in metabolic pathways (Paul and Stitt, 1993; Plaxton and Carswell, 1999), P status in plants also modifies metabolism by transcriptional regulation of several genes encoding enzymes with function in carbohydrate metabolism (Nielsen et al., 1998; Ciereszko et al., 2001a, 2001b). Regulation of gene expression typically represents more long-term metabolic adaptations (Gibon et al., 2004), and sugar-regulated gene expression is known to influence general activity of photosynthetic carbon (C) metabolism (Paul and Pellny, 2003; Thum et al., 2004) and a multitude of other aspects of metabolism and tissue development (Rolland et al., 2006). Likewise, interaction with P status mediated through gene expression is expected to influence overall C metabolism and vice versa. However, documented examples of cross-talk between sugar and P signaling are still relatively few. In soybean (*Glycine max*), sugar-induced expression of *VspB*, a gene encoding a vacuolar acid phosphatase, was repressed by $P_i$. This suggested a common mechanism of transcriptional control and a promoter domain, which mediates both sugar induction and $P_i$ repression, was identified (Sadka et al., 1994). In the same study, several other sugar-inducible genes were also shown to be repressed by $P_i$ in the absence of exogenous Suc. In tobacco (*Nicotiana tabacum*) seedlings, $P_i$ starvation promoted expression of a gene encoding the AGPase small subunit (*AGPS2*) and this effect was more prominent when Suc was added. Feeding $P_i$ to excised leaves also decreased the *AGPS2* transcript level by antagonizing the induction by Suc (Nielsen et al., 1998). In Arabidopsis, the impact of P on C metabolism has been exemplified by P starvation-induced expression of genes for β-glucosidase (Malboobi and Lefebvre, 1997), UDP-Glc pyrophosphorylase (UGPase), and the small subunit of ADP-Glc pyrophosphorylase (Ciereszko et al., 2001a, 2001b), and sugar induction of a $P_i$ transporter has been demonstrated in Arabidopsis roots (Lejay et al., 2003). Recently, we have shown for Arabidopsis that the sugar-inducible genes encoding β-amylase (*BMY1*) and chalcone synthase (*CHS*) are also induced by P deficiency and are more strongly regulated by sugars when leaf segments originate from P-starved plants. In addition, transcript levels of the P starvation-inducible genes *ACP5* (encoding an acid phosphatase), *RNS1* (encoding a ribonuclease), and *IPS1* (unknown function) increase in response to exogenously applied sugars, and supply of $P_i$ to the leaf segments reversed both P starvation-induced and sugar-induced gene expression (Müller et al., 2005). In accordance with these data, it has been shown for white lupine (*Lupinus albus*) that induction of a phosphate transporter and an acid phosphatase in roots in response to P deficiency requires either photosynthesis and phloem transport or exogenously applied sugar (Liu et al., 2005).

These studies demonstrate that $P_i$ influences sugar sensing of genes involved in C metabolism and also that sugars influence $P_i$-regulated genes with a function in P metabolism. However, the overall pattern and mechanisms behind these interactions still need to be outlined, and clearly this calls for closer examination.

The focus of this study was to test the effect of P starvation and Suc accumulation in combination. Our hypothesis was that synergistic effects on gene expression by these two major nutrients have widespread importance for the regulation of P starvation-dependent genes, but also that further interaction patterns exist representing different regulatory programs, which will be revealed by analysis of global gene expression.

Previously, we demonstrated that resupply of $P_i$ to starved plants is a useful approach to explore P starvation responses. Combination of this approach with sugar-feeding experiments makes it possible to control and change both $P_i$ and sugar levels in tissues over short time periods, and this setup has proven valuable to study the interaction of P and sugar sensing (Müller et al., 2004, 2005). This microarray study investigates the effect on transcriptional activity of $P_i$ and Suc concentrations, individually and in combination. Thus, we reveal novel interactions and disclose new candidate genes of signaling pathways. A large number of differentially expressed genes were identified for the two individual factors, P starvation and Suc. In addition, a subset of nearly 150 genes was synergistically regulated in response to both factors, and cluster analysis of these genes reveals a strong relationship between different patterns of interaction and functionality of genes.

## RESULTS AND DISCUSSION

In this study, comprehensive analysis of gene expression in response to P starvation and Suc treatment

was conducted using Arabidopsis Agilent microarrays with a total number of 21,500 genes represented. The aim was to explore cross-talk between transcriptional regulation in response to the two factors and to identify new candidate genes involved in P sensing.

### $P_i$ Concentrations and Sugar Content

Arabidopsis plants of similar size and development, but with different $P_i$ status, were obtained by first growing plants on limited P supply (0.05 mM $P_i$ in rockwool media) and then either maintaining these conditions or supplying the plants with high P concentration (4 mM $P_i$) for 1 week. Leaf segments with different $P_i$ status were then incubated with or without Suc. Quantification of P in leaf segments verified that the total P content in resupplied plants was increased about 7-fold as compared to starved plants and the level of $P_i$ was about 70-fold higher than in starved plants. As expected, leaves of P-starved plants had a higher level of sugars (3-fold) than P-supplied plants. During incubation, leaf segments remained floating, but with good contact to the solutions, and a considerable amount of exogenous Suc was taken up and metabolized to Fru and Glc (Table I). These data confirm that the samples represent different combinations of $P_i$ and sugar status.

### General Features of the P Starvation and Sugar Treatment Expression Profile

Two treatments at a time were compared on a set of slides revealing the relative changes in gene expression. With a significance level of $P < 0.001$, a total of 1,319 genes responded to P starvation (6.1% of the genes on the array), 5,479 genes responded to Suc (25.5%), and 149 genes exhibited alteration in transcript level, which revealed interaction between the two factors (0.7%). Full datasets for each of the factors P, Suc, and their interaction are available (Supplemental Tables S2, S3, and S4).

There are both distinct and overlapping groups of genes regulated in response to P starvation and Suc (Fig. 1). Five hundred and five genes were significantly regulated in response to both treatments, but independently, whereas 72 genes, which also responded to both P and Suc, showed interaction between the two factors. This group of genes will respond to either of the factors alone, but the response pattern will also depend on the other factor. In principle, the combined effect of two factors without interaction could be assumed additive or multiplicative. When testing for interaction, we have assumed a multiplicative effect of the two factors as a basis for our evaluation because this is the more stringent test. A small group of 20 genes responded in a contrasting manner to both individual factors, depending on the other factor, and, therefore, on average there is no effect and the response is exclusively interaction. For example, if P depletion induces a gene at low Suc but represses the gene at high Suc, then there is on average no effect of P and the observed responses to P at high and low Suc are considered interaction.

Restricting the observations to those genes that changed expression by more than 2-fold, a total of 187 genes were regulated in response to P starvation (Fig. 1, sum of numbers in parentheses); of these, 171 genes were induced and 16 were repressed. These may represent useful targets in molecular breeding of crops for improved performance during P starvation. The list of P-responding genes (Supplemental Table S2) contains several new potential target genes compared to previous microarray studies (Hammond et al., 2003; Wu et al., 2003; Misson et al., 2005). Using the same 2-fold ratio as cutoff criteria, Suc incubation resulted in 337 up-regulated and 307 down-regulated genes.

Tight coupling exists between $P_i$ and sugar metabolism and several studies have shown an interaction between sugar metabolism and $P_i$-regulated gene expression (Sadka et al., 1994; Nielsen et al., 1998; Ciereszko et al., 2001a, 2001b; Lejay et al., 2003; Franco-Zorrilla et al., 2004; Liu et al., 2005; Müller et al., 2005). Such interactions could rely on a secondary effect; for example, sugar added may operate as a scavenger, influencing the level of $P_i$ and thereby the expression of any P-regulated gene. Alternatively, there could be direct interaction between the regulatory mechanisms for gene expression.

These data show that a large number of genes respond to both factors. However, for most of these genes (505), the two factors show no significant interaction, suggesting that these genes respond to simple

**Table I.** *Levels of $P_i$, total P, and soluble sugars in leaf segments of P-starved or P-resupplied Arabidopsis plants*

Plants were grown for 4 weeks in inert media (rockwool) and supplied with full nutrient solution containing a limiting level of $P_i$ (0.05 mM). One-half of the plants were supplied with 4 mM $P_i$ for the last 7 d, whereas the other half remained at limiting $P_i$. At the end of the growth period, leaf segments were excised and incubated for 16 h in solutions containing 100 mM Suc or water.

| Cultivation | Incubation | P Content | | Content of Soluble Sugars | | |
|---|---|---|---|---|---|---|
| | | $P_i$ | P Total | Glc | Fru | Suc |
| | | $\mu mol\ g^{-1}\ FW$ | | $\mu mol\ g^{-1}\ FW$ | | |
| 0.05 mM $P_i$ | Water | 0.16 ± 0.09 | 2.13 ± 0.12 | 1.87 ± 0.25 | 0.29 ± 0.11 | 0.71 ± 0.18 |
| | 100 mM Suc | 0.09 ± 0.02 | 1.76 ± 0.93 | 5.22 ± 2.18 | 1.45 ± 1.17 | 5.50 ± 1.31 |
| 0.05 ± 4 mM $P_i$ | Water | 11.08 ± 2.65 | 15.92 ± 5.22 | 0.68 ± 0.35 | 0.09 ± 0.06 | 0.20 ± 0.04 |
| | 100 mM Suc | 10.76 ± 0.16 | 20.81 ± 2.90 | 7.34 ± 2.55 | 5.76 ± 2.38 | 3.32 ± 0.22 |

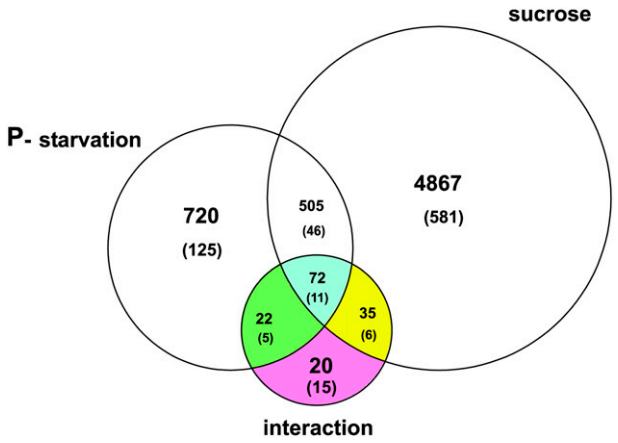


**Figure 1.** Venn diagram showing numbers of genes differentially expressed in Arabidopsis leaves in response to P starvation and Suc incubation. Isolated leaf segments of P-starved Arabidopsis plants or P-resupplied plants were incubated with or without Suc as described in Table I. Numbers in parentheses indicate genes that exhibited ≥2-fold change in expression. The colors included are used to designate groups in Figure 6.

metabolic connections. When analyzing the genes within this group that respond 2-fold or more, most of these (96%) are induced rather than repressed by sugar feeding. This is in contrast to the data for the total group of sugar-regulated genes, where 48% of the genes are repressed by sugar feeding. Thus, the group of 505 genes reacts more like the P starvation genes, where by far the majority of genes are induced (94%) rather than repressed by P starvation. We conclude that these genes, which would be characterized as sugar-sensing genes, are most likely responding to sugars indirectly through an effect on P metabolism.

Importantly, more than one-half of the P-responding genes (742) were not influenced significantly by sugar incubation. This means that most P-responding genes were still independent from sugar treatment. Thus, the experimental procedure allows for observation of interactions other than simple P scavenging. The observed levels of $P_i$ in the leaf tissues substantiate this because Suc incubation only had a limited effect on $P_i$ level even in leaves with low $P_i$ content. The experimental procedure allowed for observation of a group of 149 genes of which there is synergistic or antagonistic regulation in response to P and Suc. Of these genes, 37 showed more than a 2-fold change in expression. To our knowledge, most of these genes have not previously been described as both P and sugar responsive.

The functional categories of the genes belonging to each of the three major Venn groups—P, Suc, and interaction—were obtained from The Arabidopsis Information Resource (TAIR; www.arabidopsis.org/tools/bulk/go/index) and the relative distribution between categories was compared to the whole Arabidopsis genome. In general, only small changes in distribution were observed at this level of organization (data not shown).

### Validation of Microarray Results by Reverse Transcription-PCR

Transcriptional regulation revealed by microarrays was confirmed in a biologically independent experiment using reverse transcription (RT)-PCR (Figs. 2 and 3). Despite some quantitative differences in relative expression levels, RT-PCR data reflected the same regulatory patterns as the microarray data, showing clear induction or repression in response to Suc (Fig. 2). Similarly, induction of gene expression by P starvation was confirmed (Fig. 3), and here we also included a gene encoding a putative Tre-6-P synthase (At1g23870) that was found to be repressed in response to P starvation by both techniques. RT-PCR data confirm the expression data obtained by microarrays.

### Genes Regulated in Response to $P_i$ Starvation

In the following sections, we will mainly focus on P sensing and the interactions and less on sugar sensing because this has been covered by other studies using full Arabidopsis genome arrays and different experimental approaches (Koch, 1996; Yu, 1999; Ho et al., 2001; Lloyd and Zakhleniuk, 2004; Price et al., 2004; Thum et al., 2004; Villadsen and Smith, 2004; Loreti et al., 2005; Gonzali et al., 2006).

The genes regulated 2-fold or more in response to P starvation can be related to several functions, including P mobilization, signal transduction, transport, transcriptional regulation, and carbohydrate metabolism (Supplemental Table S3). Many of these 171 genes are directly linked to P mobilization and cleavage of $P_i$ from P-containing compounds (e.g. phosphatases and ribonucleases), which agrees with the general perception of $P_i$-dependent gene regulation (Poirier et al.,

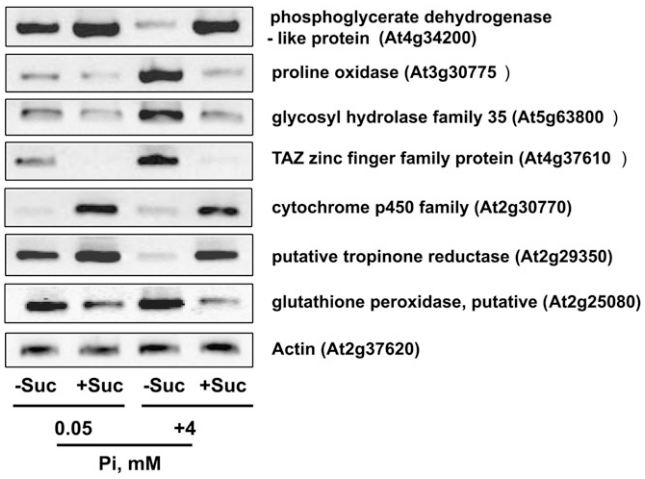


**Figure 2.** Transcript level of selected genes regulated by Suc incubation. Levels of mRNA in leaf tissue were determined by RT-PCR using gene-specific primers as listed in Supplemental Table S1. Leaf tissue was obtained as described in Table I, but in a biologically independent experiment. Leaf sections derived from plants grown on 0.05 mM $P_i$ were incubated for 16 h in solutions containing 100 mM Suc or water.

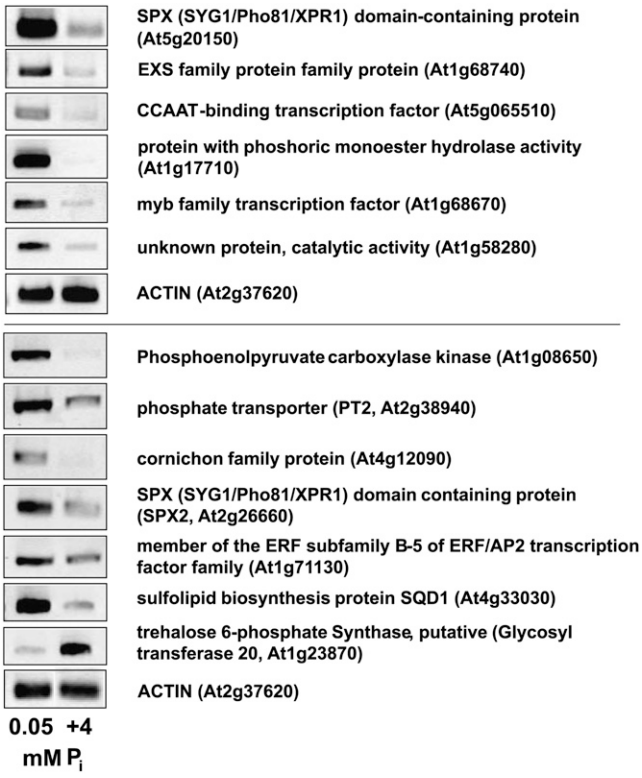


**Figure 3.** Transcript level of selected genes regulated by P starvation. Levels of mRNA were determined by RT-PCR using gene-specific primers as listed in Supplemental Table S1. Leaf tissue was obtained as described in Table I, but in a biologically independent experiment. Leaves used were derived from plants grown on 0.05 mM $P_i$ and 4 mM $P_i$, and leaf sections were incubated for 16 h in water.

1991; Vance et al., 2003). For example, the two most up-regulated genes, At1g17710 and At1g73010 (69- and 34-fold induced, respectively), appear to encode phosphatases. A high-affinity sulfate transporter (At1g22150) and a sulfolipid biosynthesis protein SQD1 (At4g33030) are also likely to contribute to P remobilization by substitution of P with sulfur (S) in lipids. Such a mechanism has been suggested for the phospholipids of thylakoid and extraplastidic membranes, which can be replaced by sulfolipids (Andersson et al., 2003) or other types of lipids (Misson et al., 2005), and the *SQD1* gene has previously been reported as a P-responsive gene (Essigmann et al., 1998; Hammond et al., 2003).

In Table II, we have listed three groups of genes being regulated by P. One important group of genes encodes $P_i$ transporters, which are classified into three families, *Pht1*, *Pht2*, and *Pht3* (Rausch and Bucher, 2002). Previous studies have demonstrated that most *Pht1* isoforms are induced during P starvation (Misson et al., 2005), but the expression pattern depends strongly on tissue localization (Muchhal and Raghothama, 1999; Mudge et al., 2002; Müller et al., 2004). In this investigation, we found several members of the *Pht1* family to be regulated, including *PHT2* (At5g43370), *PHT3* (At5g43360), *PHT4* (At2g38940), and *PHT5* (At2g32830), and one member of the *Pht3* family of mitochondrial transporters (At3g48850). The only member of the *Pht2* family was not regulated in leaves, which is consistent with previous studies (Daram et al., 1999; Müller et al., 2004).

Other regulated genes are also related to P uptake and transport. The factor, PHF1, which facilitates correct membrane location of Pht1;1 (González et al., 2005), was induced (2.7-fold) in response to P deficiency, and this might support a faster processing of transporter proteins during increased rates of synthesis. Possibly the genes encoding the SPX domain-containing proteins (At5g20150, At2g26660) and the EXS family protein (At1g68740) are also related to P transport. The SPX domain has been identified in proteins, which appear to be involved in either transport or sensing of $P_i$ (Hamburger et al., 2002; Wang et al., 2004). The SPX domain-containing proteins have some similarity to PHO1, a protein involved in xylem loading of $P_i$. The gene At5g20150 was 29-fold induced. Also, the EXS family protein contains a domain that relates to the PHO1 protein and has been classified as PHO1;H1 (Wang et al., 2004). The family comprises nine other homologs (PHO1;H2–10), but none of these responded to P starvation. Unfortunately, the *PHO1* itself was not present on the array. Finally, two genes encoding an ATP-binding cassette transporter family protein and a putative germin-like protein are also connected to transport and storage.

Another important group of P starvation-induced genes codes for enzymes active in C metabolism (e.g. key enzymes of primary metabolism, including starch degradation, glycolysis, and Suc biosynthesis). Induction of such genes is likely to promote changes in C metabolism to improve P utilization and remobilization in the cell (Plaxton and Carswell, 1999). Interestingly, a number of genes that are sugar inducible or are known to be directly related to sugar sensing were also induced by P deficiency, revealing integration of P and sugar sensing. Two examples of P-regulated genes associated with sugar sensing or C metabolism are *BMY1* (At4G15210) and *CHS* (At5g13930), which is in accordance with results by Müller et al. (2005). The expression pattern of these two genes and homologs of other genes that have earlier been reported to respond to both factors (Fig. 4) clearly demonstrates the response to both factors for *BMY1*, *CHS*, and *RNS1*, but much less for the P-responsive gene *ACP5* as also observed by Müller et al. (2005). AGPase is involved in starch biosynthesis, and three of four AGPase subunits present on the slides were induced by Suc and more so during P starvation (Fig. 4A), as also discovered for tobacco (Nielsen et al., 1998). A quite similar response was seen for two UGPase isoforms (Fig. 4B) in accordance with previous data (Ciereszko et al., 2001a, 2001b). In Figure 4, we have also included actin (At2g37620) as a reference (Fig. 4H) and five other genes (Fig. 4G) suggested to be superior as reference genes by Czechowski et al. (2005). These were all stably expressed, validating these microarray data. Among the

**Table II.** *Gene products of selected genes showing ≥2-fold change in transcript abundance in response to $P_i$ starvation and a P value <0.001 (full dataset for the selected genes in Supplemental Table S5)*

P-starved plants were produced as described in Table I. Fold changes were calculated by R as an average between the fold change when the other parameter is varied.

| AGI | Description | Fold Change | |
|---|---|---|---|
| | | Varied Factor $P_i$ | Varied Factor Suc |
| Encoding putative transcription factors | | | |
| At1g71130 | AP2 domain-containing transcription factor, putative | 4.5 | 1.6 |
| At3g05690 | CCAAT-binding transcription factor (CBF-B/NF-YA) family protein | 4.2 | 1.3 |
| At1g56650 | myb family transcription factor (MYB75) | 3.5 | 4.1 |
| At5g06510 | CCAAT-binding transcription factor (CBF-B/NF-YA) family protein | 2.6 | 1.2 |
| At1g30500 | CCAAT-binding transcription factor (CBF-B/NF-YA) family protein | 2.6 | 1.7 |
| At5g56840 | DNA-binding family protein, Myb-like DNA-binding domain | 2.6 | 0.9 |
| At1g68670 | myb family transcription factor, Myb-like DNA-binding domain | 2.4 | 1.1 |
| At3g58900 | F-box family protein contains F-box domain | 2.3 | 1.1 |
| At5g41580 | Zinc-finger (MIZ-type) family protein | 2.0 | 1.2 |
| At3g12350 | F-box family protein; similar to SKP1 interacting partner 2 | 2.0 | 1.3 |
| At2g34210 | KOW domain-containing transcription factor family protein | 2.0 | 1.3 |
| Putative signaling proteins | | | |
| At1g52940 | Calcineurin-like phosphoesterase family protein | 5.5 | 1.7 |
| At1g05000 | Tyr-specific protein phosphatase family protein | 4.4 | 3.4 |
| At4g12090 | Cornichon family protein | 4.2 | 1.3 |
| At2g32960 | Tyr-specific protein phosphatase family protein | 3.2 | 1.6 |
| At3g46120 | Calcineurin-like phosphoesterase family protein | 2.9 | 2.3 |
| At1g13750 | Calcineurin-like phosphoesterase family protein | 2.8 | 1.9 |
| At3g08720 | Ser-Thr protein kinase (PK19) | 2.4 | 1.5 |
| At1g35720 | Annexin 1 (ANN1) | 2.3 | 2.4 |
| At4g35750 | Rho-GTPase-activating protein-related | 2.3 | 1.0 |
| At4g36350 | Calcineurin-like phosphoesterase family protein | 2.2 | 1.7 |
| At5g39380 | Calmodulin-binding protein-related | 2.1 | 1.2 |
| P transporter and PHO1-like proteins | | | |
| At5g20150 | SPX (SYG1/Pho81/XPR1) domain-containing protein similar to PHO1 protein | 28.6 | 1.6 |
| At2g38940 | $P_i$ transporter (PT2) | 10.3 | 1.9 |
| At5g43360 | $P_i$ transporter (PHT3) | 7.7 | 1.9 |
| At3g47420 | Glycerol-3-P transporter | 5.2 | 1.3 |
| At1g68740 | EXS family protein/ERD1/XPR1/SYG1 family protein similar to PHO1 protein | 4.7 | 1.5 |
| At2g26660 | SPX (SYG1/Pho81/XPR1) domain-containing protein | 4.3 | 1.6 |
| At5g43370 | $P_i$ transporter (PHT2) | 3.1 | 1.1 |
| At3g52190 | Plant-specific prot. structurally related to SEC12 prot. of early secretory pathway (PHF) | 2.7 | 1.5 |
| At3g48850 | Mitochondrial phosphate transporter, putative | 2.5 | 2.6 |
| At2g32830 | $P_i$ transporter (PHT5) | 2.1 | 1.7 |

P-responding genes, we further find (expression data not shown) Suc phosphate synthase (At4g10120), a sugar transporter family protein (At1g73220), phosphoglycerate mutases (At1g22170, At1g78050), pyruvate kinase (At5g63680), a putative sugar transporter (At4G02050), phospho*enol*pyruvate (PEP) carboxylase kinase (At1g08650), putative PEP carboxylase (At1g53310), pyrophosphate:Fru-6-P phosphotransferase (PFP) $\beta$-subunit (At4g04040), and glyceraldehyde-3-P dehydrogenase (At4g34200). It is interesting to note that we find one isoform of Tre-6-P synthase induced by ample $P_i$ (verified by RT-PCR; Fig. 3) because Tre-6-P is emerging as a key regulator of plant development (Rolland et al., 2006).

Of the 187 genes regulated more than 2-fold in response to P starvation, 11 transcription factors or putative transcription factors were identified (Table II). The expression level varied among these genes, but all levels were substantially above the limit of detection, especially at the combination low P and high Suc, where expression was maximal for all genes (Supplemental Table S5). In general, there is little information on transcription factors involved in regulation during P starvation. Until now, only two transcription factors have been conclusively shown to participate in P regulation in photosynthetic eukaryotes. These are the MYB family proteins, PSR1 from *Chlamydomonas reinhardtii* (Wykoff et al., 1999; Moseley et al., 2006) and PHR1 from Arabidopsis (Rubio et al., 2001). Two more related transcription factors appear to be associated with nutrient stress responses (Todd et al., 2004), and these factors belong to a family of 15 genes with homology to PHR1. In this study, none of the genes in this family, including PHR1, responded to P starvation.

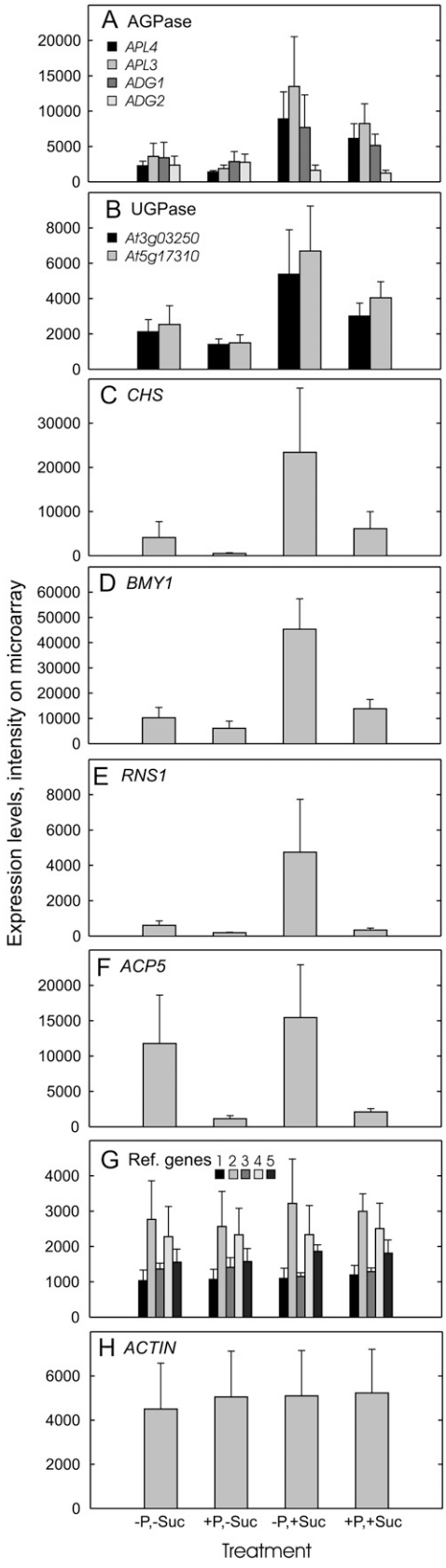


**Figure 4.** Transcript level of selected genes related to C metabolism and genes previously known to be showing regulation in response to both $P_i$ and Suc. Leaf tissue with different P and Suc content was obtained as described in Table I, and relative expression levels were analyzed by microarray as described in Figure 1. The genes in G are 1, At4g34270; 2, At1g13320; 3, At1g59830; 4, At4g33380; and 5, At2g28390.

However, tissue localization is important because we know PHR1 is regulated in roots in response to P (L. Nilsson, unpublished data). Among the 11 transcription factors that we found to be regulated in response to P starvation, three encode MYB proteins. These are interesting candidates for P-regulating factors, although they do not belong to the PHR1 family. The most regulated (4.5-fold) of the transcription factor genes encodes an AP2 domain protein (At1g71130), which belongs to a group of factors with a highly conserved DNA-binding domain. Many of these factors belong to the ethylene-responsive element-binding protein group of transcription factors, several of which are known to be responsive to environmental stresses (Weigel, 1995). The AP2 domain-containing transcription factor was also observed in a recent microarray study (Misson et al., 2005). Finally, we find 11 P starvation-induced genes encoding proteins likely to be involved in signal transduction. These include a cornichon family protein (At4g12090), a putative calcium-dependent protein kinase (At1g08650), and a putative receptor kinase (At4g31240; Table II). We further analyzed the genes listed in Table II for coexpressed gene networks using the ATTED-II database (http://www.atted.bio.titech.ac.jp), which integrates the expression pattern from a large number of microarray studies. Several genes that were highly coregulated with the genes listed in Table II were also identified as P-regulated genes in this study. For the two most regulated factors, AP2 and CCAAT-binding transcription factor (At3g05690), we found that, respectively, six and eight of the 10 most correlated genes belonged to the P-regulated genes in our study. Thus, the lists of coexpression revealed by ATTED-II also extend to expression during P starvation, which suggests that these two factors are good candidates to be directly involved in regulating a part of the P response.

Table III shows the 40 most up-regulated genes and their fold change in response to P starvation. This list includes a number of the above-mentioned genes with direct relation to $P_i$ metabolism and six genes with unknown function.

The vast majority of the genes responding to $P_i$ are up-regulated, whereas only 16 of 187 genes were down-regulated in response to P starvation (Supplemental Table S2). No common function is evident from the small selection of repressed genes. Apparently, there are only few dispensable reactions that are turned off to improve use of P in the leaf. One interesting response is the reduction of a light-harvesting complex II protein. This could represent a mechanism to reduce light harvesting to alleviate overreduction of photosystems as a consequence of $P_i$ limitation. However,

**Table III.** *Gene products of the 40 most up-regulated genes in response to P starvation and a P value <0.001 (a full dataset is listed in Supplemental Table S2)*

P-starved plants were produced as described in Table I.

| AGI | Description | Fold Change |
|---|---|---|
| At1g17710 | Similar to expressed protein (Arabidopsis; TAIR: At1g73010.1); similar to putative phosphatase (tomato) | 69.1 |
| At1g73010 | Expressed protein similar to phosphatase, orphan 1 (GI: 20196841; *Mus musculus*), (GI: 20196839; *Homo sapiens*) | 33.8 |
| At5g20150 | SPX (SYG1/Pho81/XPR1) domain-containing protein similar to PHO1 protein | 28.6 |
| At1g08310 | Esterase/lipase/thioesterase family protein contains Interpro entry IPR000379 | 20.3 |
| At1g23110 | Hypothetical protein | 19.8 |
| At5g01220 | UDP-sulfoquinovose:DAG sulfoquinovosyltransferase/sulfolipid synthase (SQD2) | 12.1 |
| At3g02040 | Glycerophosphoryl diester phosphodiesterase family protein | 11.4 |
| At2g38940 | $P_i$ transporter (PT2) identical to $P_i$ transporter (AtPT2) | 10.3 |
| At2g02990 | Ribonuclease 1 (RNS1) identical to ribonuclease SP: P42813 ribonuclease 1 precursor | 10.2 |
| At3g17790 | Acid phosphatase type 5 (ACP5) contains Pfam profile: PF00149 calcineurin-like phosphoesterase | 8.5 |
| At1g08650 | PEP carboxylase kinase identical to PEP carboxylase kinase | 8.2 |
| At2g11810 | 1,2-Diacylglycerol 3-$\beta$-galactosyltransferase, putative/monogalactosyldiacylglycerol synthase, putative/monogalactosyldiacylglycerol synthase synthase | 7.7 |
| At5g43360 | $P_i$ transporter (PHT3) identical to $P_i$ transporter | 7.7 |
| At1g58280 | Expressed protein | 7.4 |
| At4g33030 | UDP-sulfoquinovose synthase/sulfite:UDP-Glc sulfotransferase/sulfolipid biosynthesis protein (SQD1) | 6.8 |
| At3g44510 | Expressed protein | 6.5 |
| At3g05630 | Phospholipase D, putative (PLDP2) identical to SP\|Q9M9W8 phospholipase D p2 | 6.0 |
| At1g52940 | Calcineurin-like phosphoesterase family protein contains Pfam profile: PF00149 calcineurin-like phosphoesterase | 5.5 |
| At1g32740 | Expressed protein | 5.4 |
| At3g47420 | Glycerol-3-P transporter, putative/glycerol 3-P permease | 5.2 |
| At5g20410 | 1,2-Diacylglycerol 3-$\beta$-galactosyltransferase, putative/monogalactosyldiacylglycerol synthase, putative/monogalactosyldiacylglycerol synthase synthase | 5.2 |
| At4g19810 | Glycosyl hydrolase family 18 protein similar to chitinase/lysozyme GI: 467689 from tobacco | 5.1 |
| At5g20790 | Expressed protein predicted protein, Arabidopsis | 5.0 |
| At3g56040 | Expressed protein | 5.0 |
| At3g44520 | Esterase/lipase/thioesterase family protein similar to SP\|Q02104 lipase 1 precursor (EC 3.1.1.3; triacylglycerol lipase) | 4.8 |
| At1g03495 | Pseudogene, transferase family similar to anthocyanin 5-aromatic acyltransferase from *Gentiana triflora* GI: 4185599 | 4.8 |
| At1g56600 | Galactinol synthase, putative similar to galactinol synthase, isoform GolS-1 GI: 5608497 from *Ajuga reptans* | 4.7 |
| At5g39130 | Germin-like protein, putative identical to germin-like protein subfamily 1 member 16 | 4.7 |
| At1g68740 | EXS family protein/ERD1/XPR1/SYG1 family protein similar to PHO1 protein | 4.7 |
| At4g19720 | Glycosyl hydrolase family 18 protein similar to chitinase, class V GI: 899342 from tobacco | 4.6 |
| At1g71130 | AP2 domain-containing transcription factor, putative | 4.5 |
| At1g05000 | Tyr-specific protein phosphatase family protein contains Tyr-specific protein phosphatases active site | 4.4 |
| At2g34810 | FAD-binding domain-containing protein similar to SP\|P30986 reticuline oxidase precursor | 4.4 |
| At4g03960 | Tyr-specific protein phosphatase family protein contains Tyr-specific protein phosphatases active site | 4.4 |
| At2g26660 | SPX (SYG1/Pho81/XPR1) domain-containing protein low similarity to NUC-2 (*Neurospora crassa*) | 4.3 |
| At5g39160 | Germin-like protein (GLP2a) (GLP5a) identical to germin-like protein subfamily 1 member 18 | 4.3 |
| At3g12500 | Basic endochitinase identical to basic endochitinase precursor | 4.3 |
| At4g12090 | Cornichon family protein contains Pfam profile: PF03311 cornichon protein | 4.2 |
| At3g05690 | CCAAT-binding transcription factor (CBF-B/NF-YA) family protein | 4.2 |
| At5g13930 | CHS/naringenin-CHS | 4.2 |

there are several light-harvesting complex isoforms and none of the others were responding to $P_i$ treatment. Two other genes in the list are *NMT1* and *NMT3*, which encode enzymes, phosphoethanolamine *N*-methyltransferases, involved in phospholipid biosynthesis; their regulation suggests specific reduction of the formation of phospholipids, thereby complementing the mechanisms for up-regulation of alternative lipids during P starvation.

In two previous array studies, Hammond et al. (2003) and Wu et al. (2003) divided the P starvation responses into two categories, the specific responses and the general responses. Hammond found that transient changes in gene expression taking place a short time (4 h) after P withdrawal were highly variable and many nonspecific genes were induced during this time period. Opposed to this, genes up-regulated after a longer period of P deficiency (100 h) were more specific to P starvation. Wu et al. (2003) found *SEN1*, a senescence-induced gene, to be highly up-regulated in response to P starvation in both leaves and roots. Apparently, P starvation obtained by complete

**Table IV.** *Occurrence of promoter motifs in differentially expressed genes showing response to P starvation with fold change ≥2*

Upstream regions (1,000 bp) of differentially expressed genes were analyzed for the occurrence of the various motifs. The frequency in the 1,000-bp upstream regions was analyzed for the whole genome using the Patmatch tool at TAIR (http://www.arabidopsis.org/index.jsp). For references to motifs, see Rubio et al. (2001)[a]; Hammond et al., (2003)[b]; and Tang et al. (2001)[c]. Overrepresented motifs were identified using promomer at BAR (http://bbc.botany.utoronto.ca/).

| Name/Sequence | Genes Induced by P Starvation (169 Genes) | | | Genes Repressed by P Starvation (16 Genes) | | | Genome % Genes with Motif |
|---|---|---|---|---|---|---|---|
| | Genes with Motif | Motifs | % Genes with Motif | Genes with Motif | Motifs | % Genes with Motif | |
| [a]PHR1, GNATATNC | 80 | 128 | 47.3 | 2 | 3 | 12.5 | 18.1 |
| [b]P responsive, ATGCCAT | 12 | 13 | 7.1 | 0 | 0 | 0 | 4.3 |
| [b]PHO, CACGT(G/C) | 45 | 56 | 26.6 | 7 | 8 | 43.8 | 22.1 |
| [b]PHO-like, (G/T/A)(C/T/A)GTGG | 44 | 51 | 26 | 5 | 5 | 31.25 | 24.8 |
| [b]TATA box, TATAAATA | 31 | 32 | 18.3 | 2 | 3 | 12.5 | 16 |
| [b]TC-rich, TCTCTCT | 43 | 52 | 25.4 | 2 | 4 | 12.5 | 24.8 |
| [b]NIT2, TATC(A/T)(A/T) | 143 | 388 | 84.6 | 11 | 41 | 68.8 | 86.3 |
| [b]NIT2-like, AAATATCT | 14 | 14 | 8.2 | 3 | 3 | 18.8 | 8 |
| [b]HLH, CA(T/G)(A/C)TG | 89 | 128 | 52.7 | 9 | 13 | 56.3 | 49.8 |
| [c]HD-ZIP, CATTAATTAG | 0 | 0 | 0.0 | 0 | 0 | 0.0 | 0.0 |
| Overrepresented motif searches | | | | | | | |
| AGTTTT | 86 | 144 | 50.9 | 12 | 32 | 75.0 | 58.7 |
| GAATAT | 101 | 171 | 59.8 | 6 | 8 | 37.5 | 37.8 |
| ANGAATATNC | 26 | 30 | 15.4 | 1 | 1 | 6.3 | 2.6 |

withdrawal of the nutrient after high supply conditions presents a severe general stress condition to the plants. To avoid this, Misson et al. (2005) maintained a low level of P supply to the P-starved plants, but despite this precaution they observed induction of a large number of stress-responsive genes. Potentially, the exchange of the complete nutrient solution may induce the stress responses. In this study, fewer genes directly related to stress responses were induced during P starvation and this may relate to several factors: (1) longer cultivation at low P may promote adaptation to any unspecific stress conditions and subsequent supply of high P most likely does not promote stress; (2) plants were grown and incubated at low light intensities; and (3) only leaves are analyzed and any abrupt change in the nutrient solution is more likely to impose general stress on the roots. These differences in the regulation of stress-responsive genes emphasize the importance of the choice of the experimental system. Addition of $P_i$ to plants grown on low P can be done without exchanging the entire nutrient solution and will therefore impose less stress on the plants. An initial longer period of low P, as in this study, will efficiently induce genes to improve P mobilization and resupply of P to these plants will primarily reveal these specific P responses. Furthermore, the relative changes in $P_i$ levels in the leaves are much more pronounced than during starvation developing from a high P situation. Therefore, our data complement previous array studies.

### cis-Regulatory Elements in Promoters of Regulated Genes

Analyzing 1,000-bp upstream promoter regions using Athena Web tools (O'Connor et al., 2005; http://www.bioinformatics2.wsu.edu/cgi-bin/Athena/cgi/home.pl) revealed strongly significant ($P < 10^{-5}$) enrichment of four motifs (ABA response element-like binding-site motif, CACGTG motif, ACGT core of motif A in ABA response element of the rice [*Oryza sativa*] gene *Osem*, and a motif present in a group of GA

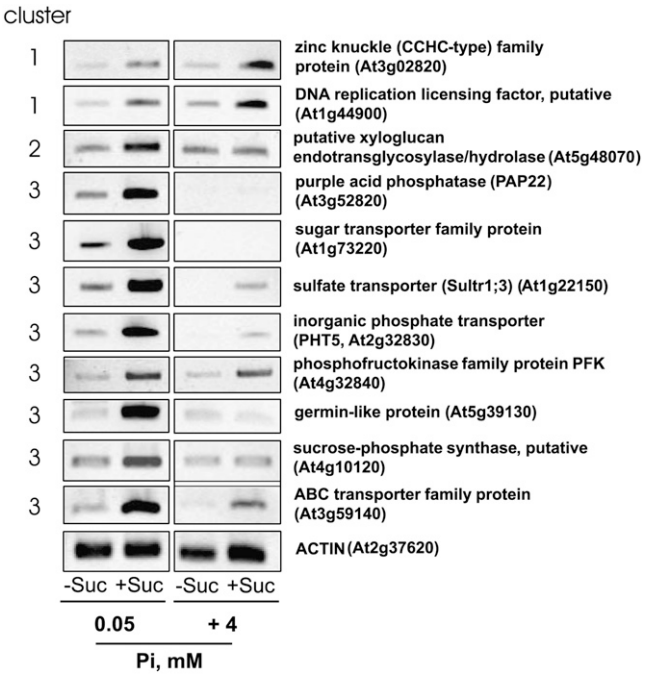


**Figure 5.** Transcript level of selected genes regulated by the interaction of P starvation and Suc. Levels of mRNA were determined by RT-PCR using gene-specific primers as listed in Supplemental Table S1. Leaf tissue was obtained as described in Table I, but in a biologically independent experiment. Cluster numbers indicate to which cluster in Figure 6 the individual gene belongs.

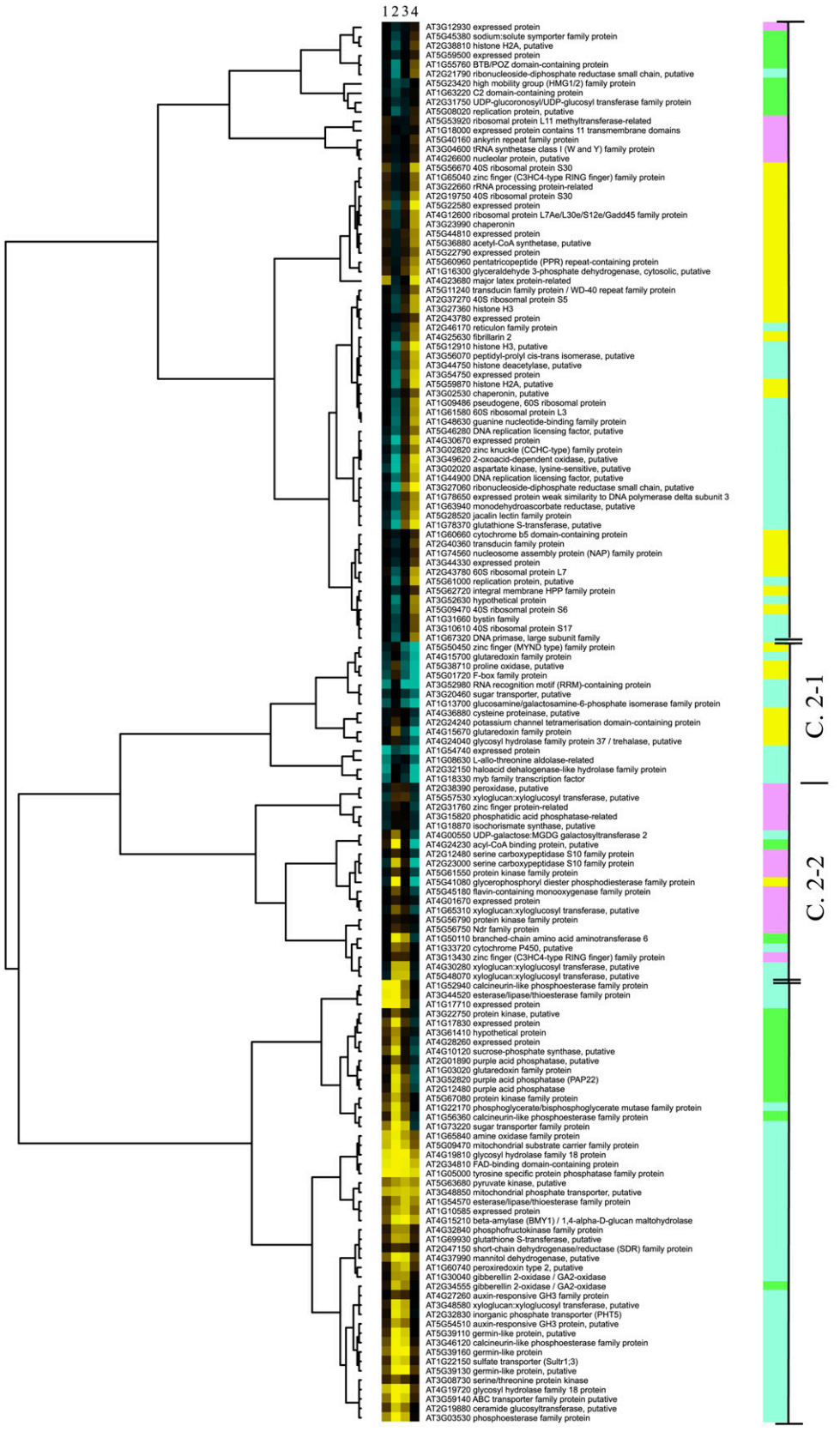


**Figure 6.** Clustering analysis of differentially expressed genes showing interaction between the two factors Suc and P starvation. Leaf tissue with different P and sugar content was obtained as described in Table I. Hierarchical clustering was performed with the

down-regulated Arabidopsis genes) in the 581 genes regulated more than 2-fold in response to Suc feeding. The same elements were also more frequent in the 46 genes regulated 2-fold by both $P_i$ and Suc. In contrast, no motifs were significantly enriched in the 125 genes regulated only by $P_i$, and analyzing all the different groups in the Venn diagram (Fig. 1) by this approach did not reveal further significantly enriched elements. However, it should be noted that Athena does not test for all known elements and, for example, the presence of the PHR1 binding site needs to be analyzed using alternative methods.

The frequency of motifs previously suggested to be mediating P responsiveness of genes was analyzed for all P-regulated genes showing more than 2-fold induction or repression by P starvation, respectively, and this was compared to the frequency in the whole Arabidopsis genome (Table IV). For induced genes, the only both frequent and considerably enriched motif compared to the entire genome was the PHR1 binding site (Rubio et al., 2001) GNATATNC. Interestingly, we also find a connection between the occurrence of the PHR1 binding site and the fold change in response to P. It is not a strict correlation, but the binding site is more frequent in the most regulated genes and, notably, the seven most regulated genes all contain two or three copies of this element (data not shown). For the repressed genes, the most enriched motif was the PHO element. However, the number of repressed genes is low (16 genes) and the observed enrichment may not be significant. All other motifs analyzed were not overrepresented and therefore do not seem to be important for P-dependent gene regulation in leaf tissue.

New potential cis-elements were searched using the promomer program (Toufighi et al., 2005; http://bbc.botany.utoronto.ca/ntools/cgi-bin/BAR_Promomer.cgi), which will identify enriched nucleotide sequences of 4 to 10 bp. One element, AGTTTT, appeared to be enriched in 16 P-repressed genes. Evaluating whether this is a true cis-element must await experimental analysis. Similar analysis of the induced genes revealed the motif GAATAT. Evaluating longer motifs and aligning all the sequences that included this core suggested that the motif could be further extended to ANGAATATNC. This motif includes the PHR1 recognition site, which validates that this unbiased approach can identify frequently occurring cis-elements. It further indicates that PHR1 might have a preference for an A in the second position of the binding site (now N) and for an additional A 2 bp upstream. Several other motifs were enriched, but these elements did not give similarly consistent results when different motif sizes were tested. Further cis-elements can potentially be revealed by more extensive approaches (e.g. as outlined by Geisler et al. [2006], who discovered several new Suc-responsive elements by an iterative method).

### Genes Regulated in Response to Suc

In plants, sugar accumulation induces the expression of genes involved in the synthesis of polysaccharides, storage proteins, pigments (e.g. anthocyanins), and genes associated with defense responses and respiration (Koch, 1996; Ho et al., 2001; Price et al., 2004). By contrast, sugar deprivation induces genes involved in photosynthesis and resource remobilization, including degradation of starch, lipids, and proteins (Yu, 1999; Price et al., 2004). This is in good accordance with our data, where the 644 genes regulated more than 2-fold (Supplemental Table S3) were most frequently related to energy, metabolism, and storage proteins (analyzed by the Classification SuperViewer at http://bbc.botany.utoronto.ca).

The three genes most strongly induced in response to Suc encode a Glc-6-P/phosphate translocator precursor (*GPT*; At1g61800) induced 8.8-fold, a *CHS* up-regulated 6.6-fold (At5g13930), and a glycosyl hydrolase family 1 protein (At1g52400) induced 6.5-fold. Normally, *GPT* is not highly expressed in photosynthetic tissue, but it can be induced by stress conditions and, in the *pho3* mutant, *GPT* is strongly induced (Lloyd and Zakhleniuk, 2004). CHS participates in the biosynthesis of flavonoids and is required for the accumulation of anthocyanins, which is known to be stimulated by Suc (Teng et al., 2005; Solfanelli et al., 2006). Induction of anthocyanin biosynthesis depends on the MYB transcription factor PAP1 (At1g56650; Tohge et al., 2005). Teng et al. (2005) found that expression of *PAP1* was induced by Suc, which is in accordance with our study where *PAP1* was 4-fold induced in response to Suc. Interestingly, P starvation applied in this investigation resulted in 3.5-fold induction of *PAP1*.

### Genes Regulated in Response to the Interaction of P Starvation and Suc

Interaction of both factors is defined as a change in expression level in response to the two factors together, which is significantly higher or lower than expected from the changes in response to the two factors applied individually. In total, 149 genes displayed a highly significant interaction between the two factors (Fig. 1). Microarray data were verified by RT-PCR for 11 of the genes showing interaction, chosen to represent diverse functionality and strong

**Figure 6.** (*Continued.*)
Cluster program (Eisen et al., 1998; http://rana.lbl.gov) based on $\log_2$ intensity ratios. Only genes revealing interaction between the two factors Suc and P starvation were included in the analysis. Blue color indicates repression and yellow color induction. Three main clusters are indicated. Treatments from left to right: 1, low/high P, no Suc; 2, low/high P, plus Suc; 3, plus Suc/no Suc, low P; and 4, plus Suc/no Suc, high P. Color code at right refers to Venn groups as indicated in Figure 1.

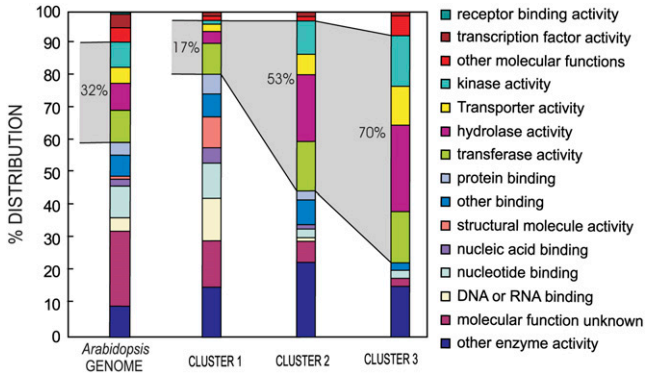


**Figure 7.** Molecular function of gene products belonging to clusters 1 to 3. Differentially expressed genes were divided into three clusters as described in Figure 6. Function of gene products was ascribed according to categorization in TAIR gene ontology annotation (www.arabidopsis.org/tools/bulk/go/index.jsp). Gene annotations corresponding to kinase, transferase, hydrolase, and transporter activities are emphasized as a group by shaded background area. In total, these represent 17%, 53%, and 70% of the gene annotations in clusters 1, 2, and 3, respectively (note that each gene can be annotated in several groups).

regulation (Fig. 5). Most of these provided clear examples of expression patterns where induction by P starvation is much stronger in the presence of high sugar level. In addition, two of the genes (At3g02820 and At1g44900) show a different pattern, with higher expression when both P and sugar are ample.

To obtain an overview of the transcriptional pattern for these genes, clustering analysis was conducted. $Log_2$ values of expression intensity ratios for each of the four comparisons were used as a basis for hierarchical cluster analysis (Eisen et al., 1998; http://rana.lbl.gov). The genes organize into three main clusters (Fig. 6), where clusters 1, 2, and 3 comprise 66, 36, and 47 genes, respectively. These genes are also listed with their expression levels in Supplemental Table S4.

Further categorization with respect to molecular function of the three clusters reveals large differences in the function of these three groups of genes (Fig. 7). The total group of 149 genes only showed minor deviation from the entire genome (data not shown), but this covers up that the interaction group includes different types of responses. When clustered according to their expression pattern, the genes also organize into different functions. Cluster 1 covers a relatively diverse group of genes, with clear overrepresentation of genes with structural molecule activity (9.5%), which were all coding for ribosomal subunits, and less frequently kinase, transporter, and hydrolase activities (7% compared to 22% for the entire genome). Cluster 1 is also enriched in DNA or RNA, protein, nucleic acid, or other binding (in total 42%), when compared to genes belonging to cluster 2 or 3. In contrast, genes belonging to cluster 2 and even more pronounced cluster 3 have a notably higher representation of kinase, transporter, hydrolase, and transferase activity. Together, these functional groups amount to 53% for cluster 2 and 70% for cluster 3, as compared to only 17% of the genes in cluster 1 (Fig. 7). To some degree, the clusters reflect which of the Venn groups (Fig. 1) the genes belong to as indicated by the color code in Figure 6.

To allow for overall comparison of expression pattern in each cluster, the level of expression was normalized for each gene, with the highest expression level set to 1 and the average expression pattern was

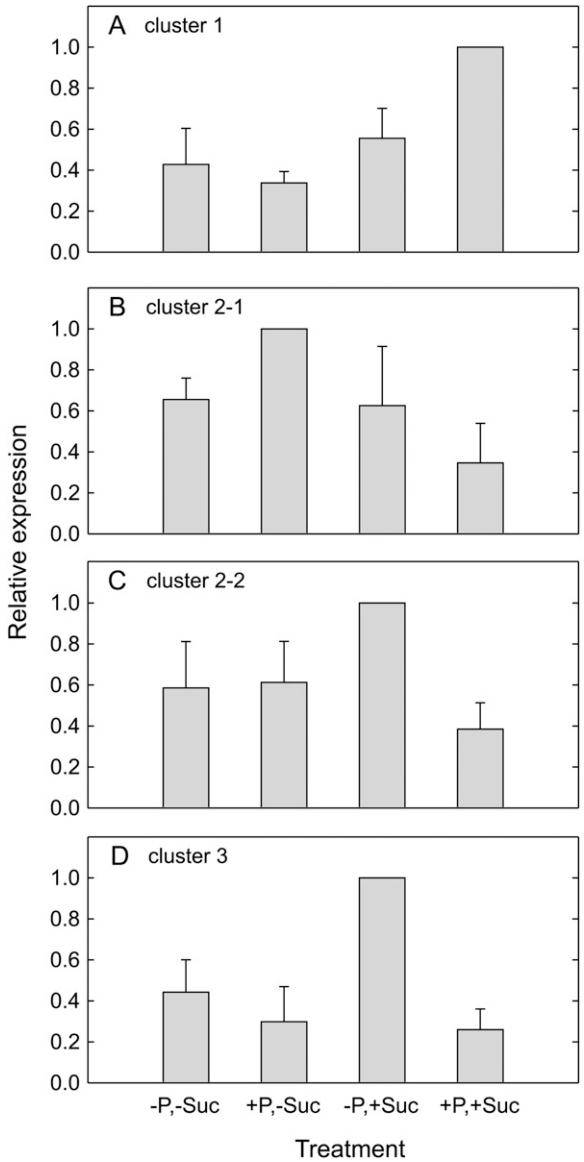


**Figure 8.** Relative transcript levels of the 37 genes regulated more than 2-fold and belonging to each of the clusters as shown in Figure 7. For each gene, the level of expression was normalized with the highest level set to 1 and the average expression pattern was plotted for the clusters. For cluster 2, plots were divided into the two major subclusters. Columns indicate mean relative expression for a group of genes and vertical bars indicate SD; number of genes included A, eight; B, four; C, seven; and D, 18.

plotted for genes that were regulated more than 2-fold (Fig. 8). For cluster 2, the two major subclusters (Fig. 6) were plotted separately because these showed a clearly different pattern. Genes in cluster 1 are induced by Suc feeding, but more so when the tissue has enough of both nutrients, P and sugar (Fig. 8A). Two examples of genes belonging to cluster 1 (Fig. 5, top images) clearly illustrate this pattern. Considering that the genes of cluster 1 are diverse in function and notably enriched in ribosomal subunits, we suggest that this cluster represents a molecular program to support increased growth and development when nutrients, in general, are ample. This agrees well with the notion that the function of many of the gene products of cluster 1 can be associated to cell growth, protein synthesis, regulation of the cell cycle, and DNA replication.

A search for overrepresented promoter motifs in cluster 1 revealed that, of 66 genes, 11 had promoter sequences that contained a short motif TCCCGC similar to the E2F binding-site motif (TTTCCCGC). The high frequency in cluster 1 contrasts with absence from clusters 2 and 3 and low frequency in the Arabidopsis genome. Table V shows enrichment of both the short motif core and the full E2F binding site. In a genome-wide search of E2F binding sites within the Arabidopsis genome, Ramirez-Parra et al. (2003) identified over 180 potential E2F target genes. Many of the targets were cell-cycle-related genes, but also other functional categories, such as transcription, stress, defense, and signaling, were found. In accordance, in our study all genes in cluster 1, which have the E2F binding site in their promoter, are cell cycle-related genes.

Genes in cluster 2 are less consistent in their regulation and fall into two subclusters that have a different expression pattern (Fig. 8, B and C). The first group has a complex pattern, being induced by P starvation at low sugar but repressed at high sugar. The other group is more related to P starvation-induced genes because these are preferentially expressed at low P in combination with high sugar.

Genes in cluster 3 are induced by P starvation and also by Suc feeding and more so at low P (Fig. 8D). The expression patterns of eight genes belonging to this cluster were also confirmed by RT-PCR (Fig. 5). This pattern is compatible with genes primarily regulated in response to P starvation, a response that is then accentuated by Suc. As expected from this pattern, cluster 3 comprises genes that are likely to be directly involved in P mobilization either by cleavage of P from P-containing compounds or by metabolic changes resulting in improved P utilization and remobilization. Examples of such genes are purple acid phosphatases (At3g52820, At2g18130), calcineurin-like phosphoesterases (At1g52940, At3g46120), and phosphoesterase family proteins (At3g03530). Indeed, most of the genes characterized as hydrolases in this cluster were phosphatases. Examples of genes coding for enzymes of carbohydrate metabolism are Suc phosphate synthase (At4g10120), *BMY1* (At4g15210), pyruvate kinase (At5g63680), and PFK (At4g32840). In this cluster, we also find transporter and storage-associated genes, such as a P transporter (At3g48850), genes coding for germins and germin-like transporters (At5g39110, At5g39160, At5g39130), a Suc transporter (At1g73220), and an ATP-binding cassette transporter (At3g59140). Induction of these genes can also be assumed to improve P metabolism of the tissue.

In cluster 3, genes encoding ethylene-responsive proteins and GA-regulated proteins were regulated in response to P starvation, and an auxin- and two GA-regulated proteins were induced in response to the interaction of both factors. In roots, regulation via these plant hormones, especially ethylene and auxin, is likely to be involved in alteration of developmental processes in response to limited P (Lynch and Brown, 1997; Wu et al., 2003). These data would suggest a P-signaling role for these hormones also in leaves. It has been shown that mitogen-activated protein kinase cascades are involved in GA, auxin, and ethylene-induced signaling, but details of the interaction between these hormone-responsive proteins and the mitogen-activated protein kinase are not clear (Wu et al., 2003). In addition, induction of genes coding for a Rho GTPase activation protein and a calmodulin-binding protein was found.

In agreement with our observation that cluster 3 represents genes induced to alleviate P starvation, we also find that the PHR1 binding site is specifically enriched in this cluster (Table V). Searching for other known or new motifs has not revealed any further motifs unique to the interaction clusters.

**Table V.** *Occurrence of P1BS and E2F in differentially expressed genes showing interaction between the two factors Suc and P starvation*

Differentially expressed genes were divided into three clusters as described in Figure 6 and 1,000-bp upstream regions for each were analyzed for the occurrence of the motifs. The frequency in the 1,000-bp upstream regions was analyzed for the whole genome using the Patmatch tool at TAIR (http://www.arabidopsis.org/index.jsp) choosing single + strand (Watson).

| Genes | No. Genes in Cluster | PHR1 Binding Site, P1BS (GNATATNC) | | | E2F Binding Site (TTTCCCGC) | | | Core of E2F Binding Site (TCCCGC) | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Genes with Motif | Motifs | % Genes with Motif | Genes with Motif | Motifs | % Genes with Motif | Genes with Motif | Motifs | % Genes with Motif |
| Cluster 1 | 65 | 8 | 9 | 12.3 | 7 | 16 | 10.8 | 11 | 24 | 16.9 |
| Cluster 2 | 36 | 10 | 13 | 27.8 | 0 | 0 | 0 | 1 | 1 | 2.8 |
| Cluster 3 | 47 | 20 | 28 | 42.6 | 0 | 0 | 0 | 1 | 1 | 2.2 |
| Genome | | | | 18.1 | | | 1.1 | | | 5.0 |

Plants will experience severe stress when limiting P is combined with conditions that promote accumulation of carbohydrates, for example, during high irradiation or low temperature. We suggest that the cooperative regulation of genes observed in cluster 3 is likely to have evolved to meet the need for alleviation of P starvation during such conditions.

Thus, organization of the genes showing interaction in three clusters reveals a close relationship between functionality of the gene products and the expression pattern. These data outline the contours of at least two regulatory programs. One, represented by cluster 1, serves to control genes with relation to growth and development during ample nutrient conditions. Another program, represented by cluster 3, has a function in coordination of adaptation to P starvation and C metabolism. A possible third program, represented by cluster 2, seems related to cluster 3 (subcluster 2-2), but, in general, cluster 2 is not as well defined.

In this study, we have identified possible cis-regulatory elements, but it has not been possible to establish any unique elements for the genes showing interaction, and this may suggest that known elements, such as the PHR1 binding site, are important also for the observed interaction pattern. We suggest that an important future research topic should be to experimentally investigate whether these coordinated genes possess separate promoter elements that can be related to sugar and P responsiveness, respectively, or whether changing one element will affect the response to both factors.

## MATERIALS AND METHODS

### Plant Material and Cultivation Conditions

Seeds of Arabidopsis (*Arabidopsis thaliana*) ecotype Columbia were germinated on soil and, after 3 weeks, transferred to 40-mL rockwool cubes (Rockwool). Plants were placed in a growth chamber at 20°C, 70% relative humidity, and 120 $\mu$mol $m^{-2}$ $s^{-1}$ photosynthetically active radiation over an 8-h photoperiod and supplied with nutrient solution with limiting $P_i$ concentration (0.05 mM) as described by Müller et al. (2004). Three weeks after transferring the plants to rockwool, one part of the plants was supplied with 4 mM $P_i$ for 1 week (+P), whereas the other plants remained on 0.05 mM $P_i$ (−P). For incubation of leaf segments, the tip and the base of each leaf were excised and the remaining middle sections were incubated on water. Sectioning of leaves started at the end of the light period. Four hours later, sections were transferred to 100 mM Suc (+Suc) solution or water (−Suc) and left floating at 20°C under dim fluorescent light (50 $\mu$mol $m^{-2}$ $s^{-1}$) for 16 h before harvest.

### Extraction and Determination of $P_i$, Total P, and Soluble Sugars

$P_i$ and total P were extracted and quantified as described by Müller et al. (2004). Soluble sugars and starch were extracted as described by Nielsen et al. (1991), and sugars were quantified by enzyme-linked reduction of $NAD^+$ as described by Beutler (1984) and Kunst et al. (1984). Assays were performed in microplates and absorbance was followed at 340 nm.

### Experimental Design and Microarray Used

The microarray Agilent Arabidopsis 2 oligo was used in this study. The array contained 22,000 probes (60mers) comprising 21,500 genes (Agilent; www.home.agilent.com), which represent approximately 80% of all Arabidopsis genes.

The experiment was designed as a two-factor experiment with P = phosphate, Suc = sucrose, resulting in four treatments: (1) +P, −Suc; (2) +P, +Suc; (3) −P, −Suc; and (4) −P, +Suc. Two treatments at a time were compared by cohybridizing differentially labeled mRNA from two samples on a set of slides. The comparisons conducted were (1) (+P, −Suc) versus (−P, −Suc); (2) (+P, + Suc) versus (−P, +Suc); (3) (−P, −Suc) versus (−P, +Suc); and (4) (+P, −Suc) versus (+P, +Suc).

The experiment included four biological replicates and four technical replicates. The technical replicates included dye swaps. Each sample represented leaf material from five to 10 plants.

### RNA Extraction and Labeling, Hybridization, and Image Analysis

RNA was isolated according to Müller et al. (2004). RNA was purified using the RNeasy kit spin columns (Qiagen). RNA concentration and quality were determined spectrophotometrically measuring $A_{260}$ and $A_{280}$.

Cyanine 3- or cyanine 5-labeled cDNA targets were synthesized with the Agilent fluorescent direct labeling kit (Agilent) using 20 $\mu$g total RNA per reaction as input. The labeled target cDNA was purified using Qiaquick PCR purification kit (Qiagen). The cyanine 3- and cyanine 5-labeled targets were combined and hybridized to the slides for 17 h at 42°C using the Agilent hybridization kit according to the manufacturer's instructions.

### Image Analysis and Data Analysis

Slides were scanned (scanner GMS418M; WG), and images were analyzed using ImaGene software (BioDiscovery).

Statistical analysis was performed with the freeware program *R*, using BioConductor packages (BioConductor, 2005). Normalization was performed by the use of the Qspline method (Workman et al., 2002).

To discriminate genes with significant changes in their expression in response to P starvation or Suc or the interaction of the two factors, two-way ANOVA was conducted (the $P < 0.001$ cutoff was chosen after evaluation of the permuted data in a Vulcano-plot [Knudsen, 2002]). Hierarchical clustering was performed as described by Eisen et al. (1998). $Log_2$ values of expression intensity ratios for each of the four comparisons listed above were used as a basis for hierarchical cluster analysis using Cluster and TreeView software (http://rana.lbl.gov). Upstream regulatory sequences were analyzed using MathInspector (Quandt et al., 1995).

### Extraction of RNA and RT-PCR

To verify the microarray data with an independent method, RNA was isolated and DNase treated, and RT-PCR was conducted according to Müller et al. (2004). cDNA was synthesized using the Bio-Rad RT-PCR kit according to the manufacturer's specifications using oligo$(dT)_{15}$ as primer. PCR primers used and size of the products are listed in Supplemental Table S1.

### Supplemental Data

The following materials are available in the online version of this article.

**Supplemental Table S1.** Primers used for RT-PCR amplification.

**Supplemental Table S2.** List of 1,319 genes differentially expressed in response to P starvation ($P < 0.001$).

**Supplemental Table S3.** List of 5,479 genes differentially expressed in response to Suc ($P < 0.001$).

**Supplemental Table S4.** List of differentially expressed genes ($P < 0.001$) showing interaction between the two factors Suc and P starvation.

**Supplemental Table S5.** Full dataset for the list of selected genes presented in Table II.

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